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(54) Title: SYNTHESIS OF STEREOSPECIFIC OLIGONUCLEOTIDE PHOSPHOROTHIOATES

(57) Abstract

Disclosed is a method for synthesizing of stereospecific (Rp) phosphorothioate oligonucleotides. In this method, a primer comprising a plurality of deoxyribonucleotides and a ribonucleotide at the 5' terminal or 5' penultimate position, is annealed to a template. The structure is contacted with a mixture of deoxynucleoside α-triphosphate Sp diastereomers and a DNA polymerase to form a PS-Rp oligodeoxynucleotide extension which is liberated as a single-stranded PS-Rp oligonucleotide by cleavage after the ribonucleotide in the primer. Also disclosed are PS-Rp oligonucleotides and oligonucleotides prepared according to this method.

SYNTHESIS OF STEREOSPECIFIC OLIGONUCLEOTIDE PHOSPHOROTHIOATES

BACKGROUND OF THE INVENTION

This invention relates to antisense oligonucleotides. More specifically, this invention relates to methods of preparing stereospecific phosphorothioate oligonucleotides.

Phosphorothioate analogs of oligodeoxynucleotides (PS oligonucleotides) are known to be useful as antisense tools (see, e.g., Agrawal et al. (1989) *Proc. Natl. Acad. Sci. (USA)* 86:7790-7794). These analogs have at least one non-bridging oxygen atom that has been substituted for a sulfur atom on the phosphate group in each internucleotide phosphate linkage. Such a modification is a conservative substitution which increases nuclease resistance without significantly impairing the hybridization of the antisense molecule with target mRNA.

PS oligonucleotides have been chemically synthesized using either the phosphoramidite or the H-phosphonate approach (both reviewed in *Methods in Molecular Biology* (Agrawal, ed.) Volume 20, Humana Press, (1993) Totowa, NJ). Such preparative methods introduce a chiral center at each internucleotide linkage that can lead to the formation of 2^n diastereomers per n internucleotide linkages, and generally result in a mixture of about 40% Rp and about 60% Sp diastereoisomers.

diastereomers of phosphorothioate oligonucleotides.

SUMMARY OF THE INVENTION

This invention provides, in one aspect, a method of synthesizing the stereospecific Rp diastereomer or isomer of a phosphorothioate oligonucleotide. As used herein, the term "phosphorothioate oligonucleotide" or "PS oligonucleotide" is intended to encompass at least five nucleotides covalently linked 3' to 5' via phosphorothioate internucleotide linkages, and including both Sp and Rp isomers. "PS-Rp oligonucleotide" refer to only the stereospecific (Rp) isomer of a PS oligonucleotide.

To produce a PS-Rp oligonucleotide according to the method of the invention, a nucleotidic primer is annealed to a complementary oligodeoxynucleotide template having 5' and 3' termini, thereby forming a partially double-stranded/partially single-stranded structure. The primer also has 3' and 5' termini and includes a nucleic acid sequence complementary to a 3' portion of the template. The primer comprises a plurality of deoxyribonucleotides and a ribonucleotide at its 3' terminal or 3' penultimate position.

The partially double-stranded/partially single-stranded structure is contacted with a mixture of deoxynucleoside α-triphosphate Sp isomers and a DNA polymerase for a time necessary

U2, RNase N1, and RNase N2, while in other embodiments, cleavage is achieved with an alkaline. In specific embodiments, a strong alkaline or base such as a halide hydroxide selected from the group consisting of sodium hydroxide, ammonium hydroxide, lithium hydroxide, or potassium hydroxide, is used for this purpose.

Another aspect of the invention are stereospecific Rp oligonucleotides prepared according to the method of the invention.

Yet another aspect of the invention includes an oligonucleotide comprising a plurality of deoxyribonucleotides covalently linked with at least one stereospecific Rp phosphorothioate internucleotide linkage. Such stereospecific Rp phosphorothioate oligonucleotides are believed to be less mitogenic than Sp phosphorothioates or than a mixture of Rp and Sp phosphorothioates. In one embodiment, all of the deoxyribonucleotides are linked with stereospecific Rp phosphorothioate internucleotide linkages.

FIG. 3B is an autoradiogram demonstrating the nuclease resistance of PO, PS-Rp, and PS oligonucleotides in the presence of T4 DNA polymerase;

FIG. 4A is a graphic representation of the hybridization affinity of PS-Rp and PS oligonucleotides to complementary DNA and RNA targets, as measured by melting temperature (T_m);

FIG. 4B is a graphic representation of the hybridization affinity of PS-Rp and PS oligonucleotides to complementary DNA and RNA targets, as measured by circular dichroism;

FIG. 5 is a graphic representation of the ability of PS-Rp (◆) and PS oligonucleotides (□) to inhibit rHIV reverse transcriptase; and

FIG. 6 is an autoradiogram demonstrating the susceptibility of PS-Rp and PS oligonucleotides to RNase H digestion.

Protocols for Oligonucleotides and Analogs, Methods in Molecular Biology, Vol. 20 (Agrawal, ed.) Humana Press, Totowa, NJ (1993) pp. 33-61). For synthesis of the primer, a plurality of deoxyribonucleotide phosphoramidites and a 3'-ribonucleotide at the position shown in FIG. 1) are incorporated with the use of a ribonucleoside phosphoramidite as the "synthon" or building block. This synthon is introduced to enable cleavage of the synthesized stereospecific oligonucleotide from the primer. After synthesis, deprotection is carried out using an RNA protocol such as desilylation (Damha et al. in *Protocols for Oligonucleotides and Analogs*, Methods in Molecular Biology, Volume 20 (Agrawal, ed.) Humana Press, Totowa, NJ (1993) pp.81-114).

According to the method of the invention, the primer is annealed to the template by methods well known in the art, such as by heating for 3 minutes at 90°C, and then cooling for one hour at room temperature. The primer is then extended to form an oligonucleotide with a nucleic acid sequence complementary to the template. Extension of the primer is carried out with any enzyme capable of such synthesis, such as Taq DNA polymerase, DNA polymerase I (Pol I polymerase), T4 DNA polymerase, and reverse transcriptase using all four 2'-deoxynucleoside 5'-(α-thio) triphosphates, or analogs of 2'-deoxynucleoside 5'-(α-thio) triphosphates, as described by Eckstein (*Ann. Rev. Biochem.* (1985) **54**:367-402).

of template, about 1 nmole of PS-Rp oligonucleotide product is obtained.

The oligonucleotides of the invention are useful as modulators of gene expression. For example, to determine the function of a particular gene, an oligonucleotide of the invention can be targeted to that gene in a tissue culture system and the effects of this modulation studied. If the object is to determine the role a particular protein plays in a certain biological process, the oligonucleotides of the invention can be used as a tool to suppress the production of the protein by targeting the nucleic acid that encodes the protein and then observing the biological effects brought about the suppression of the protein. In such a situation, it is not the integrated oligonucleotide that is being studied but the effects of suppressing production of the protein. Use of the oligonucleotides of the invention in this way provides an easily executed alternative to the laborious method of mutating the targeted gene, and thereby creating a deletion mutant.

In addition, the PS-Rp oligonucleotides of the invention, like their chiral Sp counterparts, are useful as stereochemical probes of phospho- and nucleotidyltranserase, as these oligonucleotides can distinguish between the incorporation of a deoxyribonucleotide into a growing polymer via a single bond cleavage at the phosphorus of the nucleotide triphosphate as the 3'-hydroxyl of the growing chain displaces the pyrophosphate, or via two bond cleavages if a

PS-Rp oligonucleotide and synthetic PS-oligonucleotide were also compared for their affinity for complementary DNA and RNA targets, as measured by melting temperature(T_m) and Circular dichroism. As shown in FIG. 4A, both PS-Rp and PS-oligonucleotides showed the same T_m with DNA target (51.8° and 51.9°). However, PS-Rp oligonucleotide had a higher T_m (68.9°) compared to synthetic PS-oligonucleotide (64°) with an RNA target, indicating that it has a greater binding affinity for its target. FIG. 4B demonstrates that the difference in CD spectra for RNA duplexes is more significant than that for DNA duplexes, indicating that RNA target is more sensitive to the conformational change of PS-Rp versus PS oligonucleotides at hybridization than the DNA target.

One known non-sequence-specific effect of PS oligonucleotides present as mixture of Rp and Sp diastereomers is to inhibit the enzymatic activity of reverse transcriptase. To determine whether PS-Rp isomers have the same inhibitory activity as the diasteromeric mixture, PS-Rp oligonucleotides and PS oligonucleotides were incubated individually with reverse transcriptase. As shown in FIG. 5, the PS-Rp oligonucleotide was found to be a stronger inhibitor of reverse transcriptase than the synthetic PS-oligonucleotide mixture.

The PS-Rp oligonucleotides were also subjected to RNase H digestion to determine if their ability to trigger this degradative response is comparable to that of the PS diastereomeric

thio) triphosphate (DuPont-NEN, Boston, MA). The reaction was initiated by the addition of 2500 units of sequence grade Taq DNA polymerase (Promega Corp., Madison, WI). The mixture was incubated for 4 hours at 37°C. The reaction mixture was desalted and treated with of ammonium hydroxide solution (28-30%) at 55°C for 24 hours, or with 0.3 M KOH at 37°C for 1 hour. In other tests, the desalted reaction mixture is alternatively treated with RNase A , RNase T1, RNase T2, RNase U2, RNase N1, or RNase N2, which are available from the Sigma Chemical Co. (St. Louis, MO) at 95°C for about 3 minutes. The product was dried and purified by PAGE on a 20% denaturing polyacrylamide gel. The product was excised from the gel under UV shadowing and eluted with 500 mM ammonium acetate and desalted by dialysis, as described in FIG. 1.

2. Nuclease Resistance of PS-Rp Oligonucleotides

The stability of phosphodiester-linked (PO), stereoregular (PS-Rp), and synthetic PS-oligonucleotide (PS) in bovine and human serum was tested as follows. 60 pmole of [^{32}P] 5'-end-labelled oligonucleotide was incubated in 100 ul of human serum (Sigma Chemical Co., St. Louis, MO) at 37°C. Aliquots of 20 μl were sampled at time 0, 30, 60, and 120 minutes, and then incubated with 20 μl of 20 mM Tris, pH 7.8, 10 mM NaCl, 10 mM EDTA, 0.5% SDS, and 100 μg proteinase K at 65°C for one hour. The samples were extracted by phenol/chloroform, ethanol precipitated, and then analyzed by PAGE in gels containing 20%

6 µl stop solution (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The samples were then analyzed by PAGE in a 20% acrylamide, 8.3% urea gel. The results are shown in FIG. 3B.

3. Melting Temperature Studies

Melting temperature (T_m) studies were carried as follows. 1.06×10^{-6} M PS-Rp or PS oligonucleotides were placed in 10 mM phosphate, 100 mM NaCl, pH 7, subjected to temperatures ranging from 30 to 75°C, and the absorbance at A^{260} measured using a spectrophotometer (GBC 920, GBC, Victoria, Australia). The results are shown in FIG. 4A.

4. Circular Dichroism Studies

Circular dichroism studies were performed to determine the relative binding affinity of PS-Rp and PS oligonucleotides to complementary RNA or DNA sequences. 1.06×10^{-6} M PS-Rp or PS oligonucleotides were placed in 10 mM phosphate, 10 mM NaCl, pH 7.0, and subjected to 200-320 nm UV light using a spectropolarimeter (J-710, Jasco, Easton, MD). The results are shown in FIG. 4B.

5. Inhibition of rHIV Reverse Transcriptase

In order to determine the relative ability of PS-Rp oligonucleotides to inhibit reverse transcriptase, the following assay was performed. A 10 µl reaction mixture containing 1 mM of the

was autoradiographed. The results are shown in FIG. 6.

7. Activity Assay

Activity of the PS-Rp oligonucleotides is measured by assaying their ability to inhibit HIV-1 expression in H9, MOLT-3, or other cells. In this assay, 5 x 10^5 cells per ml are infected with 2.5-5 x 10^8 virus particles of HIV-1 strains HTLV-IIIB or HTLV-IIIC. Infection with 500-1000 virus particles per cell represents a multiplicity of infection (MOI) of 0.5-1. Infection of cells is carried out by simultaneous addition of virus and antisense oligomers to the cells cultured in medium containing RPMI 1640 (10% (vol/vol) fetal bovine serum, 2 mM glutamine, and 250 μl of gentamicin per ml) in a humidified atmosphere containing 5% CO_2 at 37°C. After 4 days, the cells and supernatant are examined for the level of HIV-1 expression by measuring syncytia (MOLT-3 cells) and viral antigen expression as well as cell viability. The number of syncytia formed in MOLT-3 cells is counted after triturating the cells to obtain an even distribution of the syncytia in the culture. The average number of syncytia is obtained by counting several fields in duplicate cultures. Cell viability is measured in the presence of trypan blue, and the cells that excluded the dye are counted as viable cells. HIV-1 antigen expression is measured in cells fixed in methanol/acetone. Briefly, the cells were pelleted and then resuspended in phosphate-buffered saline (PBS) at a concentration of 10^6

NaCl, 1.5 mM $MgCl_2$) and 50 µl of 5% Nonidet P-40 (NP-40). After centrifugation at 2000 rpm for 5 min, the upper cytoplasmic fraction (combined cytosol and cell membrane) is removed and the pellet (nuclei) dissolved in 500 µl of buffer A and measured for radioactivity.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCTCGCACC CATCTCTCTC CTTCT 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA/RNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTGGCTAGC GTAGUC 16

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGAAGGAGAG AGATGGGTGC GAGAGACTAC GCTAGCCACC 40

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs

What is claimed is:

1. A method of synthesizing an oligonucleotide with stereospecific Rp phosphorothioate (PS) internucleotide linkages comprising the steps of:

(a) annealing a primer to a template, thereby forming a partially double-stranded/partially single stranded structure,

the template having a 5′ terminus and a 3′ terminus and comprising a plurality of deoxyribonucleotides, and

the primer having a 3′ terminus and a 5′ terminus and comprising a plurality of deoxyribonucleotides and a ribonucleotide at the 5′ terminus or at a 5′ penultimate position, the primer having a nucleic acid sequence complementary to a 5′ portion of the template;

(b) contacting the structure with a mixture of deoxynucleoside α-triphosphate Sp diastereomers and a DNA polymerase for a time necessary and under conditions conducive for the enzymatic synthesis of a PS-Rp oligodeoxynucleotide extension complementary to a 5′ portion of the template, thereby forming a double-stranded product;

(c) cleaving the double-stranded product after the ribonucleotide such that the PS-Rp oligodeoxynucleotide is liberated from the template and the primer.

8. The method of claim 1 wherein the ribonucleotide in the primer is at the 3' penultimate position.

9. A PS-Rp oligonucleotide prepared according to the method of claim 1.

10. An oligonucleotide comprising at least two stereospecific Rp phosphorothioates.

11. An oligonucleotide comprising a plurality of deoxyribonucleotides covalently linked with at least one stereospecific Rp phosphorothioate internucleotide linkage.

12. The oligonucleotide of claim 11 in which all of the deoxyribonucleotides are linked with stereospecific Rp phosphorothioate internucleotide linkages.

primer
5'-dGGTGGCTAGCGTAGCrU-3'
3'-dCCACCGATCGCATCG-AGAGAGCGTGGGTAGAGAGGAAGA-5'
template
deoxynucleoside α-thiophosphates
(Sp-isomers)
DNA polymerase
5'-dGGTGGCTAGCGTAGCrUdCTCTCGCACCCATCTCTCCTTCT-3'
3'-dCCACCGATCGCATCG-A-GAGAGCGTGGGTAGAGAGGAAGA-5'
primer cleavage
5'-dGGTGGCTAGCGTAGCrUp-3' and 5'-dCTCTCGACCCATCTCTCCTTCT-3' (All PS-Rp)

FIG. 1B

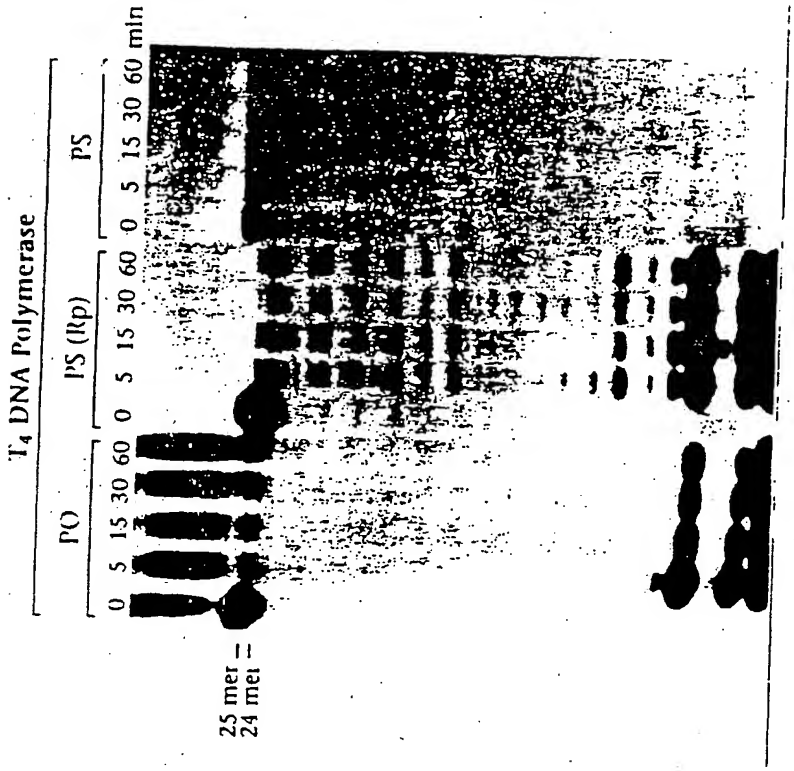

FIG. 3B

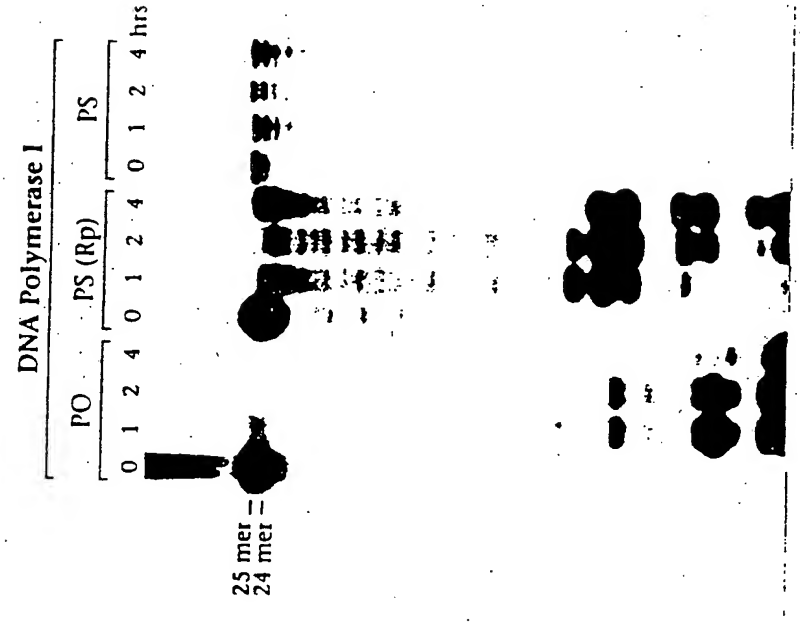

FIG. 3A

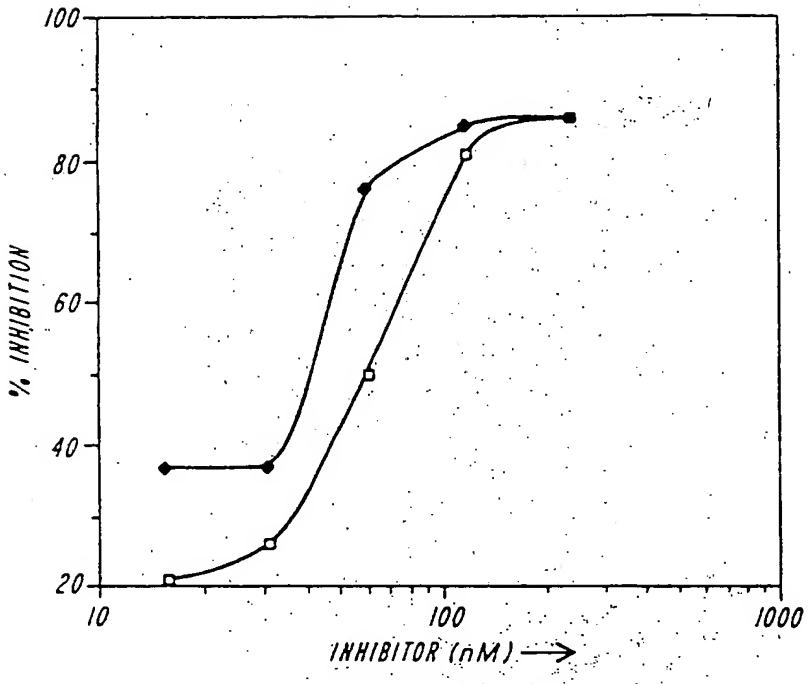

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/16086

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 08296 (ISIS PHARMACEUTICALS, INC.) 29 April 1993 see page 37; claims 14,21; examples 53,54 ---	1-12
X	NUCLEIC ACIDS RESEARCH, vol. 19, no. 3, 11 February 1991, OXFORD GB, pages 547-552, XP002001635 T.UEDA ET AL.: "Phosphorothioate-containing RNAs show mRNA activity in the prokaryotic systems in vitro" see the whole document -----	9-12

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 April 1996	10.05.96

Name and mailing address of the ISA	Authorized officer
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